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Biomarkers as Predictors in Health and Ecological Risk Assessment

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ABSTRACT

Biomarkers are measurable biological parameters that change in response to xenobiotic exposure and other environmental or physiological stressors, and can be indices of toxicant exposure or effects. If the biomarkers are sufficiently specific and well characterized, they can have great utility in the risk assessment process by providing an indication of the degree of exposure of humans or animals in natural populations to a specific xenobiotic or class of xenobiotics. Most biomarkers are effective as indices of exposure, but adequate information is rarely available on the appropriate dose-response curves to have well-described biomarkers of effect that can be widely applicable to additional populations. Specific examples of acetylcholinesterase inhibition following exposure to organophosphorus insecticides are cited from experiments in both mammals (rats) and fish. These experiments have indicated that the degree of inhibition can be readily influenced by endogenous (*e.g.*, age) and exogenous (*e.g.*, chemical exposures) factors, and that the degree of inhibition is not readily correlated with toxicological effects. Caution is urged, therefore, in an attempt to utilize biomarkers in the risk assessment process until more complete documentation is available on the specificity, sensitivity, and time course of changes, and on the impact of multiple exposures or the time of exposures.

Key Words: acetylcholinesterase; organophosphorus insecticides; exposure biomarkers; effects biomarkers; species differences.

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INTRODUCTION

Biomarkers can be generically defined in toxicology as biological parameters that reflect changes in the condition or health of an organism or population resulting from exposure to a toxicant. These will usually be discrete biochemical, physiological or histological measures that are sensitive to toxicant exposure through covalent binding to the toxicant or by a change in the level of a parameter (such as induction of activity) following exposure to the toxicant. The two best uses of biomarkers in toxicology and risk assessment are as biomarkers of exposure and biomarkers of effects. Biomarkers of exposure are indications that an organism or a population has experienced exposure to a toxicant or other stressor. However, the change in the biomarker is not necessarily related directly to the toxicant's specific mechanism of action and may not be predictive of the degree of adverse effect on the organism or population. Biomarkers of effect are specifically associated with the toxicant's mechanism of action and are sufficiently well characterized to relate the degree of biomarker modification to the degree of adverse effect. Changes in population occurrence or density as an indicator of population stress (such as a shift in natural populations to opportunistic tubifex worms in polluted environments) are more typically called bioindicators, not biomarkers.

A third type of biomarker, those of susceptibility, is of greatest use in the medical arena far predicting an individual's susceptibility to develop a given disease, (*i.e.*, genes that predispose an individual to multiple sclerosis or breast cancer). Such biomarkers could also have utility in risk assessment in identifying sensitive individuals or subpopulations that have a unique susceptibility to a toxicant's action. A human genetic polymorphism in cytochrome P450 2D6 can lead to differences in sensitivity to the antihypertensive drug debrisoquine (Smith *et al.* 1992). Genetic polymorphisms in serum A-esterase substrate specificity can lead to differences in sensitivity to several organophosphorus insecticides (Furlong *et al.* 1988). These examples could be cited as possible biomarkers of subpopulation sensitivity to xenobiotic toxicity. For the most part, however, biomarkers of susceptibility have not been characterized sufficiently to provide assistance in the risk assessment process at the present time, and will not be discussed further.

BIOMARKER UTILITY IN RISK ASSESSMENT

Huggett *et al.* (1992) have identified 13 criteria, listed below, that can be applied to biomarkers. Along with this listing is an analysis by the authors of the current paper of which of these criteria are useful in their potential employment in risk assessment as well as some of the inherent uncertainties these biomarkers possess. (1) General indicators: Some biomarkers are general indicators and only suggest stress to the population; while of potentially great use in identifying stressed populations or individuals, such biomarkers would be of limited utility in risk assessment since they would not identify the degree of stress that could be attributed to the particular toxicants undergoing the risk assessment procedures. (2) Relative sensitivity: A highly sensitive biomarker could be useful in identifying exposure situations if changes in the biomarker can be measured at earlier timepoints or at lower exposure levels than traditional toxic endpoints. It must be borne in mind, however, that sensitive biomarkers may be irrelevant to the toxic response, and basing risk

assessment calculations on a biomarker irrelevant to the biological response may lead to erroneously conservative risk assessments. (3) Biological specificity: The specificity, and therefore the utility, of a biomarker may exist only in certain species or tissues because of unique characteristics of the physiology or biochemistry of that species or tissue. (4) Chemical specificity: Biomarkers that are specific to a chemical or chemical class, or at least a very limited number of classes, will be of greatest value. (5) Clarity of interpretation: Biomarkers that respond only to the toxicants of interest and not to other changes in physiology or environment are of great use in risk assessment. It should also be borne in mind that the biomarker change *per se* may not cause toxicity, even in the toxicant's target tissue, so extrapolation to the nature or the magnitude of the biological response may be extremely difficult. (6) Time to manifestation of endpoint: A relatively short response time from exposure to biomarker change will be most useful, even though the risk assessment may need to address chronic effects, such as development of cancer, or long-term behavioral deficits. However, the onset and recovery of a biomarker change may differ from that of the biological response if any type of physiological tolerance or adaptation occurs in response to the toxicological effects; if adaptation occurs, then the biomarker is likely to show longer term changes while the biological response recovers. (7) Permanence of response: If biomarker response is too transient, it may be of limited value in risk assessment, however, even with persistent markers, the time course of change and recovery of the biomarker will need to be well understood. (8) Inherent variability (reliability): Variability can result from the organism's inherent degree of response or factors external to the organism that influence its degree of response. Similarly, population variability also occurs. If the biomarker is too susceptible to internal or external variability, it may be of little use in risk assessment. (9) Linkage to higher level effects: An ideal biomarker would correlate changes directly in a quantitative manner to the adverse effect (*i.e.*, behavioral changes, reproductive aberrations, pathology, *etc.*). If the linkage cannot be made, the biomarker will be of value only as an index of exposure and not effects. (10) Applicability to field conditions: Clearly, if the biomarker cannot be monitored accurately in real populations, it will not be useful in risk assessment. (11) Validation in the field: Field validation is obviously required before the biomarker should be put into regulatory practice. (12) Methodological considerations: The ease and reproducibility of measurements must be high, so that the biomarker can be used at various locations, by numerous laboratories, and at a realistic cost to insure widespread use of the measure. (13) Status of method's utility: The biomarker must be sufficiently established and documented to give a large data base indicating consistency and reliability; this will inspire confidence among scientists that the biomarker results are meaningful.

At present the most promising biomarkers investigated rely on the induction or increase of certain molecular endpoints in response to xenobiotic or stressor exposure. Examples of biomarkers induced by xenobiotic exposure include xenobiotic metabolizing enzymes (cytochrome P450 and Phase II enzymes such as glucuronosyl transferases and glutathione transferases), stress proteins (heat shock proteins, metallothioneins, and heme and porphyrins (Stegeman *et al.* 1992)). Some of these biomarkers are reasonably specific for the xenobiotics to which they respond [*i.e.*, cytochrome P450 1A (CYP 1A) is induced by polycyclic aromatic hydrocarbons,

polychlorinated biphenyls and dioxin, and metallothionein is induced by cadmium and zinc] whereas others, such as the stress proteins, are induced by a variety of adverse conditions. The specificity and time course of biomarker protein synthesis has been widely studied in a variety of species (Stegeman *et al.* 1992). Several of these biomarkers, such as the induction of the synthesis of Phase II (conjugating) enzymes or stress proteins, seem to be protective, and are probably not reflective of the mechanism of toxicity of the xenobiotic in question. Therefore, these xenobiotics cannot serve as biomarkers of effect. Induction of the hepatic synthesis of the egg yolk precursor protein, vitellogenin, in egg-laying vertebrates by estrogenic xenobiotics reflects a portion of the pathway of the mechanism of toxicity, and therefore has the potential to serve as a biomarker of effect.

CHOLINESTERASE INHIBITION AS A BIOMARKER

This paper will concentrate on one biomarker, cholinesterase inhibition, which has been used in several contexts: an index of worker exposure to anticholinesterase insecticides; a subject of study in basic and applied research programs to characterize the biological effects of anticholinesterase insecticides in a variety of experimental animals; a possible field index of contamination of natural populations by anticholinesterase insecticides; and an index of anticholinesterase insecticide exposure and/or effect in the regulation of these insecticides. Cholinesterase inhibition meets most of the criteria cited above for suitability as a biomarker. However, there are also a number of difficulties with attempting to use cholinesterase inhibition in risk assessment, and these difficulties will also be pointed out.

Use of cholinesterase inhibition as a biomarker is predicated on the fact that the primary biochemical effect of anticholinesterase insecticides is the inhibition of acetylcholinesterase in nervous tissue in both the target insects and non-target species. This action is true of both the organophosphorus (OP) and carbamate insecticides; this paper will concentrate on the OP insecticides. The OP insecticides are presently the most widely used group of insecticides world wide. They exhibit a wide range of acute toxicity levels in mammals, with LC50 values ranging from the low mg/kg level to the g/kg level (Meister 1989; Worthing and Walker 1987). Additionally, they display a similar wide range of acute toxicity levels in lower vertebrates, but not necessarily the same rank order of toxicity levels as is observed in mammals (Mayer and Ellersieck 1986; Meister 1989; Worthing and Walker 1987).

The OP insecticides or their active metabolites are potent and persistent inhibitors of serine esterases, a group of hydrolases that contain serine in their active sites. Among these serine esterases is the widely occurring target enzyme, acetylcholinesterase (AChE), which hydrolyzes the important neurotransmitter acetylcholine to guarantee that its actions remain transient. Despite misconceptions by many, the inhibition of AChE per se is not overtly harmful. Adverse consequences of exposure to anticholinesterases result from the subsequent accumulation of acetylcholine in cholinergic synapses and neuromuscular junctions, leading to hyperactivity within these pathways. A variety of signs of autonomic and somatic system dysfunction will manifest into clinical signs including salivation, lacrimation, urination, defecation, tremors, and respiratory distress at high dosages in mammals. In the case of a lethal dose, death results from respiratory failure primarily from bronchoconstriction and

depression of brain stem respiratory control centers. Because of the involvement of cholinergic systems within the central nervous system, effects in higher brain function (*i.e.*, cognition) are possible. A delayed peripheral neuropathy is also demonstrated by a select group of OP compounds, which is not mediated by cholinesterase inhibition. Numerous studies have been conducted on the effects of OP insecticides in mammals, and the reader is referred to the following references as an initial summary: Abou-Donia (1981), Ballantyne and Marrs (1992), Chambers and Levi (1992), Ecobichon (1996), Eto (1974), and Gallo and Lawryk (1991).

While the clinical scenario of OP insecticide toxicity is well characterized in mammalian systems, the events leading to lethality in fish are not as readily characterized. Some of the OP insecticides are highly toxic to fish (Mayer and Ellersieck 1986) and can cause death in a very short period of time, but the precise organ systems targeted are not well understood. Personal observations of several fish species have indicated that OP insecticide-exposed fish become hyperactive, exhibit loss of schooling behavior, overreact to stimuli, display "piping" (gasping for air) at the water surface, and eventually become apparently incapacitated with a limited amount of prominent opercular activity ("pilling"). They can also display spinal curvature, presumably from the intense muscle spasms. The presence of an entirely different type of respiratory system in fish compared to mammals makes projections from the mammalian symptomatology to the piscine difficult, at best, so it is not possible to conclude that fish die of respiratory system failure in the same prominent sense that mammals do.

One of the useful characteristics of OP insecticides has been the "built-in" biomarker associated with the ability of the OP insecticides or their metabolites to persistently inhibit serine esterases. As mentioned above, the **critical** event in OP compound toxicity appears to be the inhibition of AChE in target tissues, which results in acetylcholine accumulation. AChE in non target tissues (such as red blood cells) can also serve as a biomarker. Other serine esterases are additional targets of inhibition. These include two esterases of unknown physiological function: (1) butyrylcholinesterase (BChE; previously called pseudocholinesterase) that preferentially hydrolyzes butyrylcholine compared to acetylcholine, occurs prominently in the plasma of many mammalian species, including humans, and has routinely been used as a biomarker in human occupational/accidental exposure situations and in laboratory animal experiments, and (2) carboxylesterases (aliesterases) that are widely distributed and hydrolyze a variety of carboxylic esters. The inhibition of either BChE or carboxylesterases does not seem to result in any adverse effects.

SELECTED UNCERTAINTIES ASSOCIATED WITH CHOLINESTERASE INHIBITION USE AS A BIOMARKER

The inhibition of AChE or possibly other serine esterases appears to be a likely parameter to be considered as a biomarker useful in the OP insecticide risk assessment process. A large data base currently exists on the inhibition of AChE from a variety of compounds in several species using numerous *in vitro* and *in vivo* experimental paradigms. Nevertheless, there is still an enormous amount of uncertainty regarding the magnitude and time course of inhibition resulting from exposure to different species, ages and compounds, and also uncertainty regarding the extent

of inhibition that exerts meaningful toxicological effects. These uncertainties will limit the utility of cholinesterase inhibition in a risk assessment procedure. Some observations made in our laboratories regarding AChE inhibition patterns in mammals (rats) and fish (channel catfish and mosquitofish) in both *in vitro* and *in vivo* experiments will be presented below to illustrate some current points of knowledge as well as current areas of uncertainty.

In mammals, the active metabolites (oxons) of several OP insecticides have been shown to be potent *in vitro* inhibitors of rat brain AChE with a range of potencies displayed (Chambers *et al.* 1990). Carboxylesterases in rat liver homogenates have also been shown to be highly sensitive (in most cases) to these same oxons, and were usually more sensitive to inhibition than brain AChE; an exception was methyl paraoxon, which was a weak inhibitor of both AChE and carboxylesterases, with the sensitivity of AChE being greater. These *in vitro* observations allowed predictions of inhibition patterns of the two enzyme activities in an *in vivo* exposure, and these predictions were borne out in *in vivo* experiments (Chambers and Carr 1993). These experiments have also illustrated that AChE inhibition and recovery is faster following administration of the oxons, whereas AChE inhibition is slower to peak and is more prolonged following administration of the parent insecticides, showing the requirement for bioactivation. While carboxylesterases are, in many cases, more sensitive to OP compound inhibition than is AChE, and could be suggested as a more sensitive biomarker, it should be emphasized that a non-target enzyme will not reflect the biochemical lesion that is responsible for the adverse effects and therefore could lead to a biomarker that is irrelevant toxicologically to the target of interest. These results showing different rates of inhibition and recovery among compounds illustrate the need to know the time course of esterase inhibition in order to properly assess the time to peak inhibition and the persistence of that inhibition.

Recent experiments in our laboratories have illustrated that juveniles displayed a greater degree of inhibition than adults when exposed to the same dosage of insecticide, reflecting the less efficient detoxication systems present in the juvenile (Atterberry *et al.* 1997). Age-related differences in response to OP insecticides have also been reported by others (Brodeur and DuBois 1963; Gaines and Linder 1986; Pope *et al.* 1991). The prior administration of the P450 inducer phenobarbital, slowed the time course of brain AChE inhibition following parathion administration, suggesting a more efficient induction of detoxication activities than activation activities by phenobarbital (Chambers and Chambers 1990). These few examples illustrate that the extent and time course of biomarker changes will vary among compounds and physiological conditions; therefore, the use of inhibition of AChE in risk assessment must be done with full knowledge of the experimental situation used in deriving the data.

Another area of uncertainty from mammalian experiments is the degree of inhibition required to elicit toxicological effects. In a limited number of behavioral experiments our laboratory has conducted using a shuttle avoidance paradigm, we found greater levels of brain AChE inhibition than observed behavioral deficits (Chambers *et al.* 1988). Behavioral deficits (using a schedule controlled experimental paradigm) were worse from very high-dose exposures to paraoxon if the effects were not antidoted centrally with atropine (Chambers and Chambers 1989). At

lower dosages there was no clear threshold of brain AChE inhibition yielding behavioral deficits in the schedule controlled design, and the performance recovered before the brain AChE activity did (Carr and Chambers 1991). Sheets *et al.* (1997) observed a lack of consistent correlations among cholinesterase inhibition and clinical signs from six OP insecticides administered in a 90-day experiment. These types of observations illustrate the current uncertainty of the degree of AChE inhibition, either centrally or peripherally, which correlates with any concurrent toxicological effects. At this time, because of this lack of correlation, any use of AChE inhibition as a biomarker in risk assessment should be used only as a biomarker of exposure and not effects. If, and when, the inhibition and adverse effects resulting from a given compound are sufficiently documented, it may be possible to consider AChE inhibition as an adverse effect in risk assessment.

Insecticide acute toxicity levels are not predictable within chemical group among vertebrate species of several classes (Chambers and Carr 1995). In comparative studies of rat and channel catfish *(Ictalurus punctatus)* brain homogenates, chlorpyrifos-oxon was found to be a more effective AChE inhibitor than paraoxon in both species (Straus and Chambers 1995; Carr and Chambers 1996), despite the fact that chlorpyrifos is less toxic to mammals than is parathion (Meister 1989; Worthing and Walker 1987). This brain AChE sensitivity difference to the oxons corresponds to the fact that chlorpyrifos is more toxic to fish than is parathion (Mayer and Ellersieck 1986). Experiments with mosquitofish *(Gambusia affinis),* which included methyl parathion (a weak toxicant in fish), along with parathion and chlorpyrifos, also showed correspondence among the potencies of the three oxons for brain AChE inhibition and the toxicity level of the parent insecticide, but no correspondence was seen in the metabolism of the insecticides (Boone and Chambers 1997). We have concluded previously that the acute toxicity levels of OP insecticides in mammals, in contrast to fish, are determined more by differences in metabolic efficiencies than by differences in target enzyme sensitivity (Chambers *et al.* 1994; Ma and Chambers 1995; Chambers and Carr 1995).

Spontaneous reactivation was not observed *in vitro* in catfish brain AChE assays, whereas reactivation was seen in rat brain AChE (Carr and Chambers 1996). This inability of fish brain AChE to spontaneously reactivate is probably the reason behind the very prolonged AChE inhibition observed following exposures of catfish or mosquitofish to OP insecticides (Carr *et al.* 1995; Straus and Chambers 1995; Boone and Chambers 1996). In these studies, high levels of AChE inhibition was observed (exceeding 90%) without mortality, and the inhibition was maintained for up to 2 weeks or longer. Additional laboratory experiments have shown inhibition of brain and muscle AChE in mosquitofish for 6 weeks (Figures 1 and 2). During an accidental contamination of a natural pond with chlorpyrifos, recovery of mosquitofish brain AChE recovered by 45 days after the exposure but muscle AChE activity did not show appreciable recovery during the 60-day observation period (Carr *et al.* 1997). During this same environmental exposure, the mosquitofish in the pond survived whereas the blue gill sunfish did not, but the brain AChE percent inhibition was greater in the mosquitofish than the blue gills. The bass and golden shiners of the pond also died, but the percent AChE inhibition in brain was similar in magnitude to that of the mosquitofish. These results indicate no simple correspondence between AChE inhibition and the likelihood of survival. These laboratory and

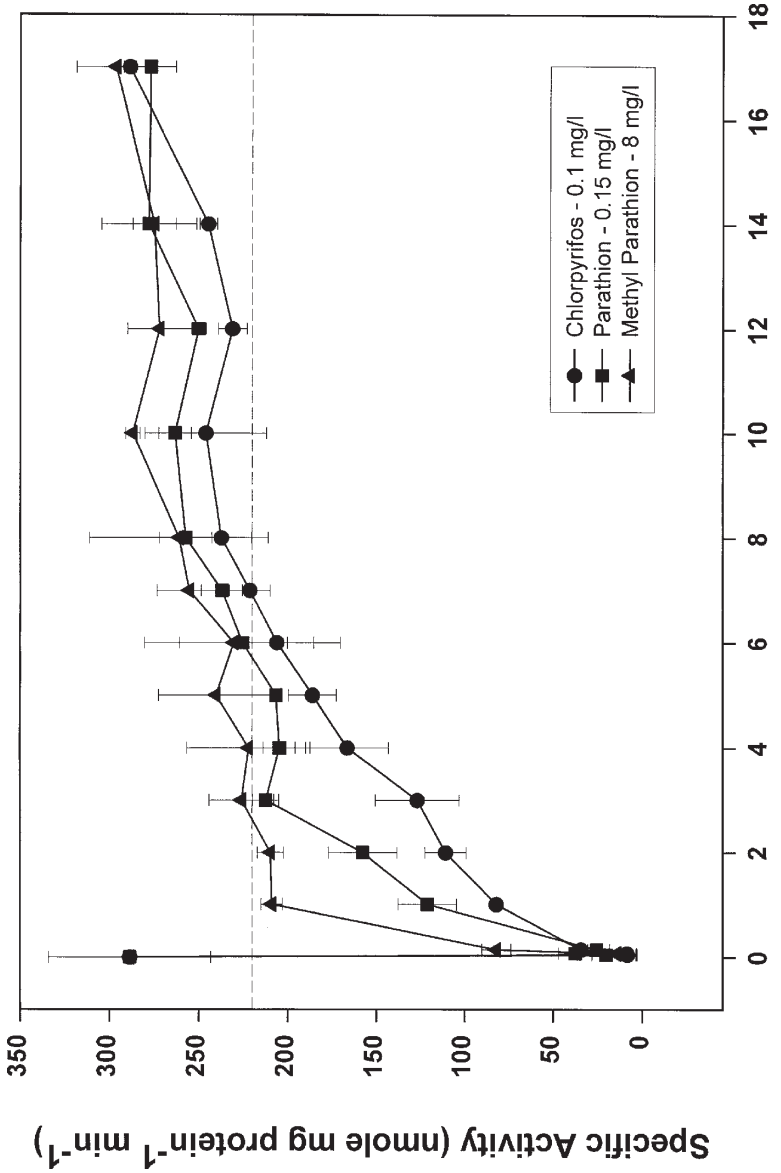

Figure 1. **Specific activity of brain acetylcholinesterase from mosquitofish (*Gambusia affinis*) exposed *in vivo* for eight hours in a static system to three organophosphorus insecticides dissolved in acetone.** Fish were exposed in 15 liter glass aquaria with 1 ml of solution per liter. Fish were not allowed to eat for 12 hours prior to or during the exposure. At eight hours post-exposure the fish were returned to a flow-through system (250 ml/min) and were allowed to eat. Values represent the mean ± S.E.M. (4 replications for each treatment time and group and 26 replications for control group). Values below the dotted line are significantly different from controls ($P \leq 0.05$, ANOVA, SNK).

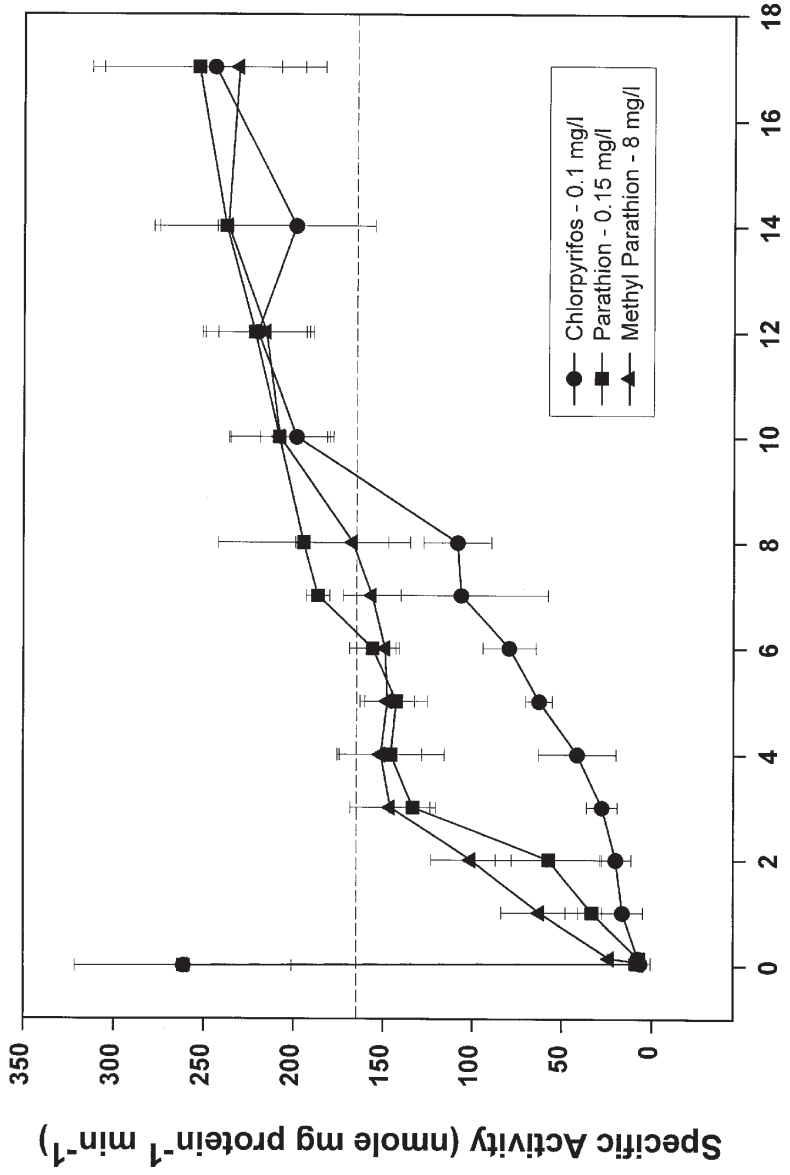

Figure 2. **Specific activity of muscle acetylcholinesterase from mosquitofish exposed *in vivo* to three organophosphorus insecticides.** Experimental conditions as described in Figure 1. Values represent the mean ± S.E.M. (4 replications for each treatment time and group and 26 replications for control group). Values below the dotted line are significantly different from controls ($P \leq 0.05$).

field observations indicate that fish can withstand high levels of AChE inhibition for extended periods of time with apparently little effect on physiological function. These results raise the question of how important brain or muscle AChE inhibition is to OP insecticide toxicity in fish. If the role of brain or muscle AChE inhibition is equivocal in OP insecticide toxicity in fish, then the use of brain or muscle AChE inhibition as a biomarker would be of less value than it could be in mammals.

CONCLUSION

In conclusion, the use of AChE inhibition can be a very useful biomarker of OP insecticide exposure; however, the dynamics of the pattern of inhibition and recovery needs to be thoroughly understood in the species of interest in order to use the biomarker in any quantitative sense. This biomarker may be of less utility in fish because of tolerance of high levels of AChE inhibition for extended periods of time. The uncertainties and data gaps regarding AChE inhibition by different compounds in different species and in individuals of different physiological condition places several caveats and conditions on the use of AChE inhibition in risk assessment at this time, and caution is urged on the use of too simplistic a view of AChE inhibition in risk assessment.

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